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Nutrient deprivation induces the Warburg effect through ROS/AMPK-dependent activation of pyruvate dehydrogenase kinase

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ABSTRACT

The Warburg effect is known to be crucial for cancer cells to acquire energy. Nutrient deficiencies are an important phenomenon in solid tumors, but the effect on cancer cell metabolism is not yet clear. In this study, we demonstrate that starvation of HeLa cells by incubation with Hank's buffered salt solution (HBSS) induced cell apoptosis, which was accompanied by the induction of reactive oxygen species (ROS) production and AMP-activated protein kinase (AMPK) phosphorylation. Notably, HBSS starvation increased lactate production, cytoplasmic pyruvate content and decreased oxygen consumption, but failed to change the lactate dehydrogenase (LDH) activity or the glucose uptake. We found that HBSS starvation rapidly induced pyruvate dehydrogenase kinase (PDK) activation and pyruvate dehydrogenase (PDH) phosphorylation, both of which were inhibited by compound C (an AMPK inhibitor), NAC (a ROS scavenger), and the dominant negative mutant of AMPK. Our data further revealed the involvement of ROS production in AMPK activation. Moreover, DCA (a PDK inhibitor), NAC, and compound C all significantly decreased HBSS starvation-induced lactate production accompanied by enhancement of HBSS starvation-induced cell apoptosis. Not only in HeLa cells, HBSS-induced lactate production and PDH phosphorylation were also observed in CL1.5, A431 and human umbilical vein endothelial cells. Taken together, we for the first time demonstrated that a low-nutrient condition drives cancer cells to utilize glycolysis to produce ATP, and this increases the Warburg effect through a novel mechanism involving ROS/AMPK-dependent activation of PDK. Such an event contributes to protecting cells from apoptosis upon nutrient deprivation.

1. Introduction

Normal tissues produce energy through catabolizing glucose via oxidative phosphorylation; however, cancer cells utilize glycolysis (i.e., the Warburg effect) to produce ATP in aerobic conditions. This shift of glucose metabolism from oxidative phosphorylation to glycolysis, although inefficient for energy production, meets the demands of rapidly proliferating cancer cells [1]. Most importantly, proliferating cancer cells can utilize intermediates of the glycolytic pathway as precursors for synthesizing amino acids, nucleic acids, and lipids, and thus meet the bioenergetic and biosynthetic demands of proliferation [2]. In addition, the Warburg effect provides several growth advantages for cancer cells; it provides the ability to survive in conditions of fluctuating oxygen levels [3], it favors tumor invasion [4], and it suppresses anticancer immune factors [5].

The glycolysis pathway involves many enzymes. Pyruvate kinase M2 (PKM2) and PDH are the two enzymes that were found to lead metabolites of glycolysis to proceed to oxidative phosphorylation or to convert to lactate by the enzyme, LDH. Pyruvate kinases have many isoforms, and the M2 type is selectively expressed in proliferating cells including cancers. Tyrosine phosphorylation of PKM2 by phosphotyrosine signaling downstream of a variety of cell growth signals reduces PKM2 activity [6]. Pyruvate produced by the low activity of PKM2 is further catabolized to lactate by LDH. High activity of PKM2, however, is associated with decreased cellular lactate production, and increased oxygen consumption [6,7]. On the other hand, PDH is one of the molecules in the PDH complex which controls

Abbreviations: AMPK, AMP-activated protein kinase; DN, dominant negative; WT, wild type; C.C, compound C; DCA, dichloroacetate; G6PDH, glucose-6-phosphate dehydrogenase; Glc, glucose; GLUT4, glucose transporter 4; HBSS, Hank's buffered salt solution; HIF, hypoxia-inducible factor; LDH, lactate dehydrogenase; PDH, pyruvate dehydrogenase; PDK, pyruvate dehydrogenase kinase; PEP, phosphoenolpyruvate; PFK, phosphofructokinase; PHD, prolyl hydroxylase; PKM2, pyruvate kinase M2; ROS, reactive oxygen species; TCA, tricarboxylic acid

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pyruvate entry into the tricarboxylic acid (TCA) cycle and thus sits at the interface between glycolysis and glucose oxidation. PDH converts pyruvate to acetyl-CoA in mitochondria, which in turn synthesizes oxaloacetate to citrate which enters the TCA cycle to generate NADH and FADH2. PDK was recently identified as a hypoxia-inducible factor (HIF)-1α-regulated gene, and is the only well-known enzyme to phosphorylate the E1 subunit of PDH at Ser293. Phosphorylation of PDH by PDK leads to PDH complex inactivation, and under this condition, pyruvate is catabolized to lactate by LDH [8,9].

Increased aerobic glycolysis and oxidative stress are important features of cancer cell metabolism [10]. Conditions associated with tumorigenesis such as hypoxia, matrix detachment, mitochondrial dysfunction, inflammation, and starvation can all lead to excess production of ROS [11–15]. ROS are important signaling molecules which control glycolysis activity. In skeletal muscles, exogenous H_2O_2 increases glucose uptake, lactate production, hexokinase (HK), phosphofructokinase (PFK), and glucose-6-phosphate dehydrogenase activities, as well as gene expression of glucose transporter 4, HKII, and PFK [16]. In addition, ROS were shown to amplify HIF-1α stabilization by inhibiting prolyl hydroxylase [17]. Furthermore, ROS are reported to induce AMPK activity [18,19]. AMPK, an energy sensor, is activated when cellular ATP is insufficient. Once AMPK activity increases, energy-consuming pathways such as lipogenesis are inhibited, while energy-producing pathways such as glucose uptake and fatty acid oxidation are upregulated [20]. Although AMPK is known to phosphorylate PFK2 and increase glycolysis [21,22], its role in regulating Warburg effect still remains to be discovered.

Nutrient deprivation widely exists in solid tumors because of the poor blood supply [23,24]. To date, the role of nutrient deprivation in cancer cell metabolism, however, has not been fully elucidated. Thus, in this study, we explored the influence of nutrient deprivation on the Warburg effect in various cell types, involving signaling pathways, and its impact on cell viability. As a result, we demonstrated that nutrient deprivation by removing all amino acids and serum can enhance aerobic glycolysis which delays cell apoptosis, and also for the first time showed that PDK is a novel target for the AMPK-mediated glycolytic response.

2. Materials and methods

2.1. Cell culture

HeLa and A431 cells were obtained from the American Type Culture Collection (Manassas, VA, USA), and CL1.5 was provided by Dr. Zhixin Yang (National Taiwan University, Taipei, Taiwan). Mouse embryonic fibroblasts (MEF) after immortalization were cultured in Dulbecco's modified Eagle medium complete medium supplemented with 10% (v/v) heated-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Human umbilical vein endothelial cells (HUVEC) were purchased from ScienCell (Carlsbad, CA, USA). Cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air.

2.2. Reagents

HBSS (containing 5 mM glucose, 1.26 mM $CaCl_2$, 0.493 mM $MgCl_2$, 0.407 mM $MgSO_4$, 5.33 mM KCl, 0.441 mM KH_2PO_4, 4.17 mM $NaHCO_3$, 137.93 mM NaCl, and 0.338 mM Na_2HPO_4), dichlorodihydrofluorescein diacetate ($DCFH_2$-DA), 2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (2-NBDG), and MitoSoxRed were obtained from Invitrogen (Rockville, MD, USA). zVAD-fmk, compound C, and bafilomycin A1 were from Calbiochem (San Diego, CA, USA). Butylated hydroxyanisole (BHA), N-acetyl-cysteine (NAC), dichloroacetate (DCA), propidium iodide (PI), and other chemicals were obtained from Sigma Aldrich (St Louis, MO, USA). Annexin V-PE and 7-aminoactinomycin D (7-AAD) were obtained from BioVision (Milpitas, CA, USA). The anti-LC3 polyclonal antibody was from MBL (Woburn, MA, USA). Specific anti-phospho-PDH E1 (Ser293) was obtained from NOVUS Biologicals (Littleton, CO, USA). Specific antibodies for phospho-AMPK (Thr172), phospho-PKM2 (Tyr105), AMPK, caspase-3, PARP, PDK1, PDH, and PKM2 were purchased from Cell Signaling Technology (Beverly, MA, USA). Mito-TEMPO was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Recombinant human PDHA1 which is a member of PDH complex and can be phosphorylated by PDK on Ser293 was obtained from SignalChem (Richmond, BC, Canada). [γ-^{32}P]ATP was purchased from NEN (Boston, MA, USA). Wild type (WT) and dominant negative (DN) AMPK plasmids were gifts from Dr. Kelly A. Wong (Whitehead Institute for Biomedical Research, Cambridge, MA, USA). The dual-tagged GFP–mRFP–LC3 plasmid was obtained from Addgene (Cambridge, MA, USA).

2.3. ROS detection

After starvation for the indicated time periods, cells were incubated in PBS containing 10 μM $DCFH_2$-DA or 5 μM MitoSoxRed for 30 min, and then washed with PBS, and immediately submitted to a flow analysis using a FACScan flow cytometer (Becton Dickinson, NJ, USA). Data were analyzed with the CellQuest program.

2.4. Annexin V/7-AAD staining

After starvation for the indicated time periods, cells were collected and washed with ice-cold PBS, and then incubated in calcium-containing PBS with Annexin V and 7-AAD for 30 min. After incubation, cells were immediately submitted to a flow analysis using a FACScan flow cytometer.

2.5. Cell cycle determination

After starvation for the indicated time periods, HeLa cells were collected and washed with ice-cold PBS, and then fixed with 70% (v/v) ethanol overnight at −20 °C. Fixed cells were washed with PBS, and then stained with 80 μg/ml PI. The cell cycle was measured by a FACScan flow cytometer.

2.6. Glucose uptake assay

2-NBDG is a fluorescent-labeled glucose analog that is incorporated into cells and allows quantification of glucose uptake. 2-NBDG was co-treated with HBSS starvation for 1 h, and the excess 2-NBDG was removed by washing with PBS. Cells were immediately submitted to a flow analysis using a FACScan flow cytometer.

2.7. ATP, LDH, pyruvate, lactate, and oxygen consumption assays

ATP was detected with a CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Madison, WI, USA). Cytosolic LDH activity was determined by manual instructions of the CytoTox 96® Non-Radioactive cytotoxicity assay (Promega). Concentrations of intracellular total and cytoplasmic pyruvate and lactate in the culture medium were respectively determined with a Pyruvate Assay Kit and Lactate Assay Kit (BioVision, Milpitas, CA, USA). For cytoplasmic pyruvate assay, starved HeLa cells were swelled in Buffer A (0.05 mM PMSF, 10 mM HEPES, 1.5 mM $MgCl_2$, 100 mM KCl and 0.5 mM DTT) for 10 min and cell membrane was broken by dounce homogenizer. To remove mitochondria, cell lysate was centrifuged at 15,000 ×g for 30 min. The supernatant was determined as cytosol fraction. For oxygen consumption, HeLa cells were seeded in 3.5-cm dishes. After the indicated treatment, cells were collected, and the oxygen consumption rate was measured with MitoCell (MT200, Strathkelvin Instruments, North Lanarkshire, Scotland).

2.8. Immunoblot analysis

Cells were lysed in lysis buffer. After sonication, protein concentrations were determined using the Bio-Rad protein assay. Equal amounts of soluble protein were electrophoresed on 8%–12%

SDS-PAGE, and transferred to Immobilon-P membranes. Nonspecific binding was blocked with 5% nonfat milk. After immunoblotting with the first specific antibodies, membranes were washed and incubated with an HRP-conjugated secondary antibody. Protein was detected with an enhanced chemiluminescence detection reagent.

2.9. In vitro PDK kinase assay

To evaluate kinase activity of PDK, total protein extracts from the stimulated cell lysates were pre-cleaned at 4 °C for 1 h and then immunoprecipitated with 1 μg of the anti-PDK1 antibody for 4 h. Afterwards 10 μl protein A-agarose beads were added and rocked for another 30 min at 4 °C. The immunocomplexes were washed with cold lysis buffer, and then with kinase reaction buffer (20 mM Tris–HCl at pH 7.5, 10 mM $MgCl_2$, 2 mM DTT, and 10 μM ATP). Beads were incubated at 30 °C for 30 min in 25 μl kinase reaction buffer supplemented with 10 μCi of [γ-^{32}P]ATP and 1 μg recombinant human PDH protein. Reaction products were run on SDS-PAGE and transferred to polyvinylidene difluoride membranes, followed by autoradiography.

2.10. Microscopic detection of autophagosome

The tandem fluorescent-tagged LC3 construct (tfLC3) was transfected to cells by means of MicroPorator (Promega, USA). After HBSS starvation for 2 h, cells were fixed with 4% paraformaldehyde and examined by confocal microscopy (ZEISS, LSM 510 META Confocal Microscope). The yellow and red puncta represent the formation of autophagosome and autolysosome, respectively.

2.11. Statistical evaluation

Values were expressed as the mean ± S.E.M. of at least three independent experiments, which were performed in duplicate. An analysis of variance (ANOVA) was used to assess the statistical significance of the differences, and p values of <0.05 were considered statistically significant.

3. Results

3.1. Nutrient deprivation induced the Warburg effect through the PDK/PDH pathway

In order to determine the effect of HBSS starvation on the Warburg response, the concentrations of lactate in the culture medium, the end product of glycolysis and the most important indicator of the Warburg effect, were detected. As shown in Fig. 1A, the extracellular level of lactate was significantly enhanced by HBSS starvation at 0.5–12 h in HeLa cells. Considering that implanted tumor cells react differently to starvation condition [25] and this effect might be specific to cancer cell types, we tested various cancer cells and normal cells. We found that HBSS can also induce lactate production in A431, CL1.5, and HUVEC, but not in MEF (Fig. 1A). In contrast, HBSS starvation significantly reduced oxygen consumption in HeLa cells (Fig. 1B), suggesting that HBSS starvation not only induced the Warburg effect but also reduced mitochondrial oxidative phosphorylation. To determine if HBSS starvation exerts any effects on metabolic pathways responsible for the increased Warburg effect, glucose uptake, intracellular LDH activity, and pyruvate content were determined. Considering the status of different glucose concentrations containing in HBSS (i.e. 5 mM glucose) and DMEM (i.e. 25 mM glucose), low glucose DMEM (5 mM) was also compared in glucose uptake assay to avoid an artificial effect from competition. As shown in Fig. 1C, HeLa cells uptake more glucose in HBSS than DMEM with 25 mM glucose; however, similar level of glucose uptake was observed in HBSS and DMEM with 5 mM glucose. We conclude that nutrient deprivation does not enhance glucose uptake within 1 h. In addition, LDH activity was not affected by nutrient deprivation (Fig. 1D). In the aspect of pyruvate, although the total amount of intracellular pyruvate was not changed by HBSS, the cytoplasmic pyruvate level was increased under nutrient deprivation (Fig. 1E).

Moreover, we also determined levels of PKM2 and PDH, two major enzymes which control the metabolism of pyruvate through either mitochondrial oxidative phosphorylation or the lactate pathway. As shown in Fig. 2A, HBSS starvation rapidly induced PDH E1 (Ser293) phosphorylation which is an inactivate form of PDH within 15–120 min, while PKM2 phosphorylation was not affected. Similar results of PDH phosphorylation by HBSS were also observed in A431, CL1.5 and HUVEC, but not in MEF (Fig. 2B). Since PDK is the only well-known enzyme to phosphorylate PDH, we tested the effect of a PDK inhibitor. We found that DCA, a PDK inhibitor [26,27], significantly inhibited HBSS starvation-induced PDH phosphorylation (Fig. 2C). To understand if increased PDH phosphorylation results from the activity change of upstream kinase PDK, we first examined its protein expression. As a result, PDK expression was not affected under HBSS treatment (Fig. 2A). To further confirm if HBSS starvation affected PDK activity, an in vitro kinase assay was conducted by immunoprecipitating PDK followed by incubation with the recombinant PDHA1 protein. As shown in Fig. 2D, PDK activity following HBSS starvation increased within 2 h. All these results suggest that nutrient deprivation can induce the Warburg effect through the PDK/PDH pathway.

To further understand what nutrient component deficiency is involved in the HBSS-induced Warburg effect, we supplemented glucose, amino acids and/or FBS in HBSS. As a result, we found that 25 mM glucose delayed HBSS-induced PDH phosphorylation, while 10% FBS had no significant effect on PDH phosphorylation. Among the 5 amino acids tested (glutamine, leucine, arginine, histidine and cysteine), only cysteine significantly inhibited HBSS-induced PDH phosphorylation (Fig. 2E).

3.2. Nutrient deprivation activates AMPK to induce Warburg effect

Since nutrient deprivation is a condition of an energy crisis, we wondered if AMPK, an intracellular energy sensor, is involved in the HBSS starvation-induced Warburg effect. First, we determined if HBSS can induce AMPK (Thr172) phosphorylation. As shown in Fig. 3A, AMPK phosphorylation was rapidly induced by HBSS starvation at 15 min and lasted for more than 2 h. Second, to understand if the induced AMPK phosphorylation is correlated with energy levels, the intracellular ATP amount was measured in HBSS-starved HeLa cells. As expected, the ATP level gradually decreased with time (Fig. 3B). Third, to understand if activated AMPK is responsible for energy conservation upon nutrient depletion, the AMPK inhibitor compound C and AMPK-DN were tested. We found that compound C and AMPK-DN further enhanced intracellular ATP loss after HBSS starvation (Fig. 3B).

Next, we were interested to determine if AMPK is involved in the HBSS starvation-induced Warburg effect. As shown in Fig. 3C, compound C alone unexpectedly induced moderate PDH phosphorylation, and under this condition, HBSS starvation-induced PDH phosphorylation could not be further detected. These results suggest that AMPK plays a role in PDH phosphorylation induced by HBSS starvation. To confirm this notion, gene manipulation of AMPK activity was conducted by overexpressing AMPK-WT or AMPK-DN. As shown in Fig. 3D, AMPK-WT and AMPK-DN alone did not have significant effects on basal PDH phosphorylation. AMPK-DN significantly reduced HBSS starvation-induced PDH phosphorylation, while AMPK-WT did not have such effect. To understand if inhibition of PDH phosphorylation by compound C and AMPK-DN is functionally associated with the outcome in energy regulation, lactate production was measured. As shown in Fig. 3E, compound C and AMPK-DN indeed inhibited HBSS starvation-induced lactate production. All these results suggest that the HBSS starvation-induced Warburg effect is mediated by AMPK activation.

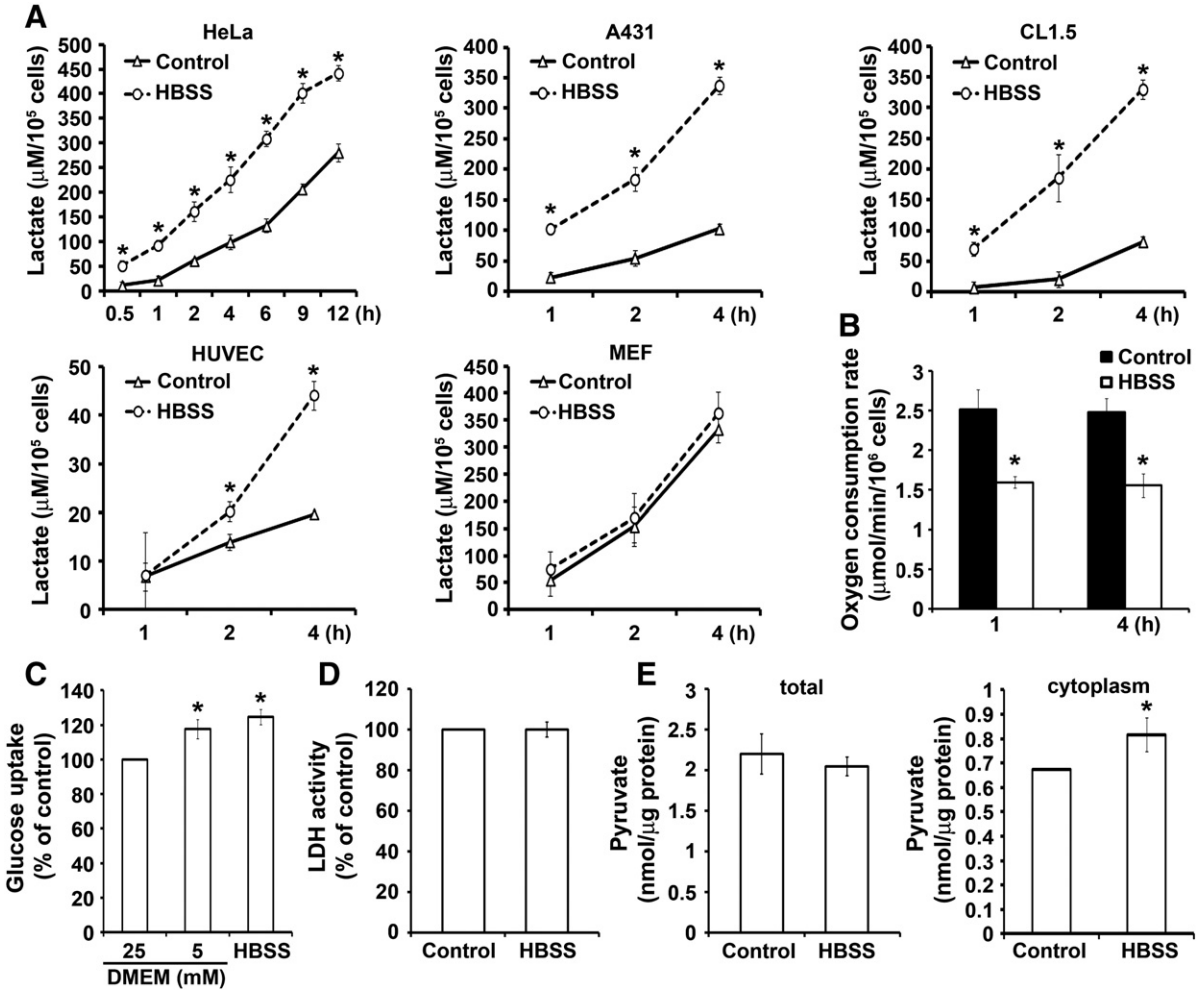

Fig. 1. Nutrient deprivation induces the Warburg effect. (A) Extracellular lactate levels in HeLa, A431, CL1.5, HUVEC and MEF, and (B) oxygen consumption in HeLa cells were detected after cells were treated with HBSS for the indicated time periods. (C) Glucose uptake, (D) LDH activity, and (E) total and cytoplasmic pyruvate were detected after HeLa cells were treated with HBSS for 1 h. Data were the mean ± S.E.M. from at least three independent experiments. * $p<0.05$, indicating significant effects of HBSS starvation and low glucose (5 mM).

3.3. ROS production induced by nutrient deprivation promotes Warburg effect and is an upstream signal of AMPK

Since ROS are known to induce AMPK activation [18,19], we explored if ROS are involved in the HBSS starvation-induced Warburg effect and are the upstream molecule for AMPK activation. First, $DCFH_2$-DA and MitoSoxRed staining were respectively used to detect cytosolic and mitochondrial ROS. We found that HBSS starvation induced a rapid but mild increase in cytosolic ROS at 0.5–4 h, which was then gradually reduced after starvation for 8 h (Fig. 4A). In contrast to cytosolic ROS, the level of mitochondrial ROS slowly increased, and achieved a marked and sustained response at 8–24 h (Fig. 4A). Next, we found that NAC which reduced cytosolic ROS increase (Fig. 4B) can significantly inhibit both basal and HBSS starvation-induced lactate production (Fig. 4C) as well as PDH phosphorylation (Fig. 4D). These results suggest that the HBSS starvation-induced Warburg effect is mediated by ROS production.

After observing that both ROS and AMPK are involved in the HBSS starvation-induced Warburg effect, we next determined their causal relationships using a pharmacological approach. Results revealed that NAC significantly inhibited HBSS starvation-induced AMPK phosphorylation (Fig. 4E). In contrast, inhibition of AMPK phosphorylation by compound C and AMPK-DN had no significant effect on cytosolic ROS production (Fig. 4F). These data indicated that ROS are the upstream signal for AMPK activation upon HBSS starvation. Finally, since HBSS starvation induced PDK-dependent PDH phosphorylation, we were interested in exploring if the ROS/AMPK signal affects PDK activation. As shown in Fig. 4G, inhibition of AMPK and ROS by compound C and NAC respectively significantly inhibited HBSS starvation-induced PDK activity. Thus, we concluded that the HBSS starvation-induced Warburg effect acts through the ROS/AMPK/PDK/PDH pathway.

3.4. The Warburg effect reduces cell apoptosis upon nutrient deprivation

Next, we were interested in clarifying the role of increased Warburg effect in cell viability under nutrient deprivation stress. First, we characterized the death phenomenon of apoptosis in HBSS starvation. We found that HBSS starvation significantly induced apoptosis of HeLa cells (Fig. 5A), and this action was inhibited by zVAD, a pan-caspase inhibitor (Fig. 5B). In addition, HBSS starvation time-dependently increased the cell population at the sub-G_1 phase at 6–24 h (Fig. 5C). To verify if HBSS starvation induced caspase-dependent cell death, immunoblotting was conducted to analyze caspase-3 cleavage. As a result, caspase-3 was cleaved to an active form after HBSS starvation for 8 h (Fig. 5D). Consistent with caspase-3 activation, PARP-1, a substrate of caspase-3, was time-dependently cleaved after HBSS starvation (Fig. 5E).

It has been reported that in dead cells MitoSoxRed can be released from the mitochondria and bind to nuclear DNA, giving a large artificial signal [28]. To clarify if HBSS-induced mitochondrial ROS production at 8–24 h (Fig. 4A) is because of cell death, excluding signal of annexin V-positive dead cells and inhibition of cell death

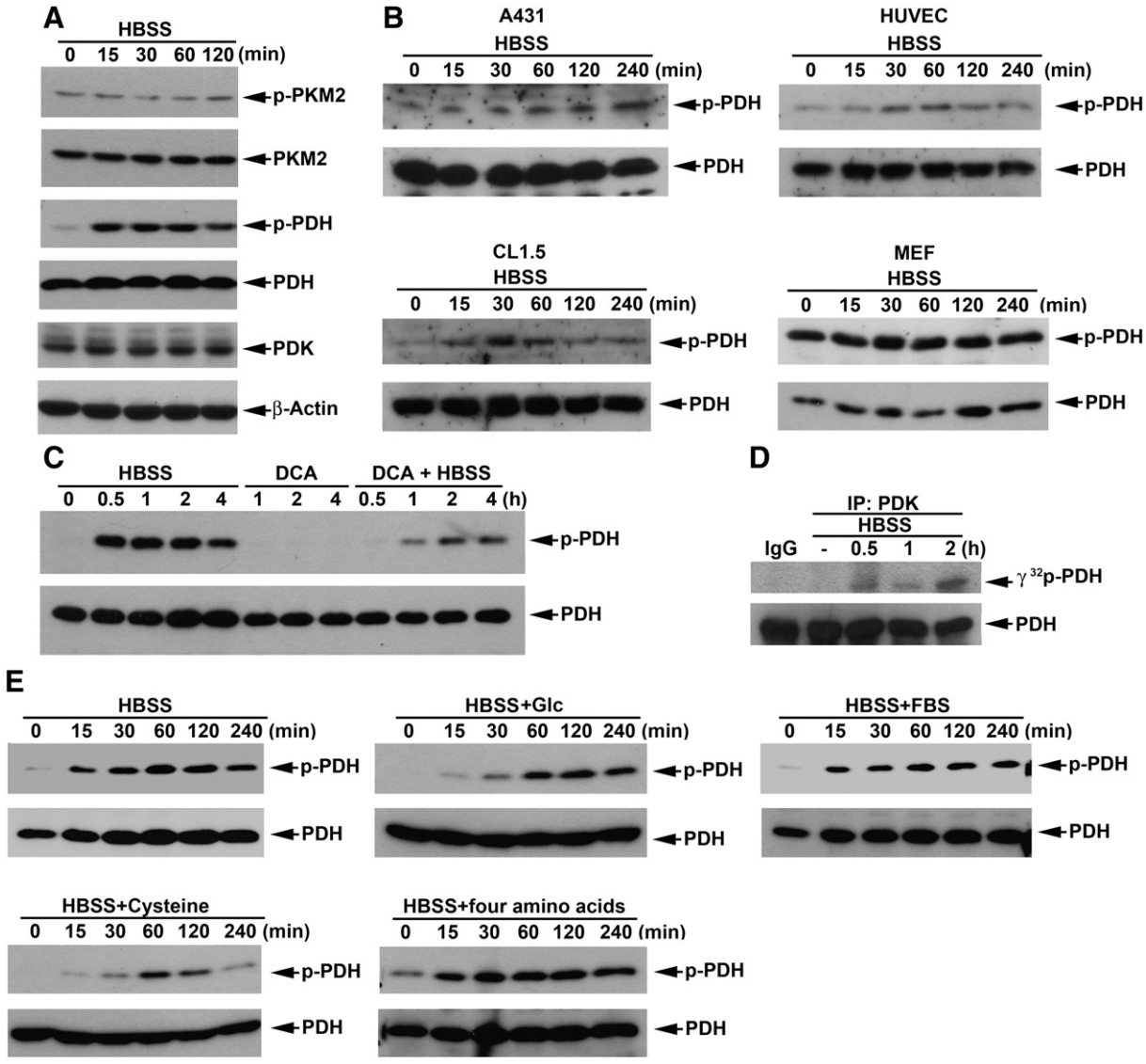

Fig. 2. Nutrient deprivation activates the PDK/PDH pathway. (A, C, E) HeLa cells and (B) A431, CL1.5, HUVEC and MEF were treated with HBSS for the indicated time periods, either in the presence or absence of DCA (10 mM), glucose (25 mM), 10% FBS, four amino acids (4 mM glutamine, 0.8 mM leucine, 0.4 mM arginine, 0.2 mM histidine) or 0.2 mM cysteine. Total cell lysates were collected and subjected to Western blot analysis using the indicated antibodies. (D) After treatment with HBSS for the indicated time periods, PDK was immunoprecipitated and subjected to kinase assay. Results were representative of three independent experiments.

by zVAD were conducted. As shown in Fig. 5F, HBSS still can induce significant mitochondrial ROS production in annexin V negative cells and zVAD-treated cells. These results suggest that HBSS indeed can increase mitochondrial ROS production at the later time point.

Next, we would like to understand if ROS/AMPK/PDK/PDH pathway is involved in the regulation of cell viability. Thus, we tested the effects of pharmacological inhibitors of ROS, AMPK and PDK on HBSS starvation-induced cell apoptosis. Results of Fig. 5G showed that DCA, a well-known inhibitor of PDK and thereby the Warburg effect, can concentration-dependently enhance HBSS starvation-induced cell apoptosis. Similarly, the antioxidants BHA, NAC and Mito-TEMPO (a mitochondrial specific ROS scavenger), which can respectively inhibit HBSS-induced cytosol ROS (Fig. 4B) and mitochondrial ROS (Fig. 5H), and compound C also enhanced HBSS starvation-induced cell apoptosis at 12 h (Fig. 5I). Notably, BHA, NAC and compound C treatment alone induced moderate cell apoptosis. These results suggest that HBSS starvation-induced ROS production and AMPK phosphorylation play a protective role in cell viability. In addition, similar to the effect of compound C, AMPK-DN expression significantly enhanced HBSS-induced cell apoptosis at 12 h (Fig. 5J). Taken together, these results suggest that the HBSS starvation-induced Warburg effect can delay cell death.

3.5. PDK is not involved in nutrient deprivation-induced autophagy

Autophagy was found to be induced when cells suffer from nutrient and energy deprivation, and can delay cell death by recycling protein and organelles to amino acids which support energy production. Since ROS increase induced by Earle's balanced salt solution, a low nutrient buffer similar to HBSS, leads to autophagy [15], and AMPK was also evidenced to induce autophagy by inhibition of mTOR [29], we wondered if PDK activity is involved in HBSS starvation-induced autophagy. First we determined HBSS starvation-induced autophagy by Western blotting and confocal microscopy. Results of Fig. S1A showed a higher signal of LC3-II than that of LC3-I in resting HeLa cells, and after inhibition of lysosome activity by bafilomycin A1, the LC3-II level significantly and time-dependently accumulated within 1–6 h. Under bafilomycin A1 treatment, the increased LC3-II/LC3-I ratio induced by HBSS was higher than normal medium. We also used a dual-tagged GFP–mRFP–

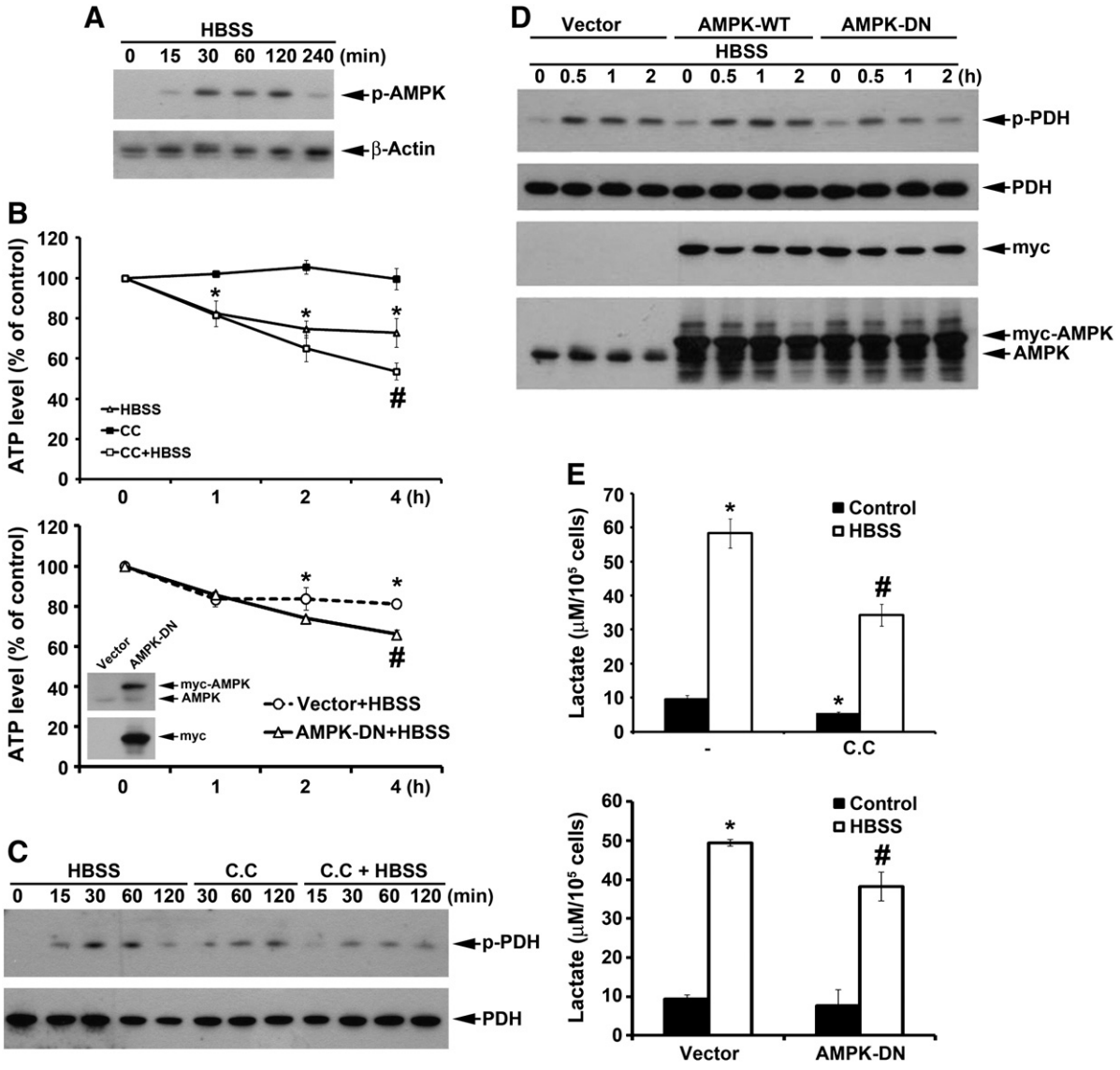

Fig. 3. AMPK is involved in the nutrient deprivation-induced Warburg effect. (A) HeLa cells were treated with HBSS for the indicated time periods, and AMPK was determined by immunoblotting. (B) After pretreatment with 10 μM compound C or overexpression of AMPK-DN, cells underwent HBSS starvation, and intracellular ATP levels were determined at the indicated time points. (C) Cells were pretreated with compound C followed by HBSS starvation. PDH phosphorylation was determined by immunoblotting. (D) Cells were transfected with myc-tagged AMPK-WT or AMPK-DN, followed by HBSS starvation. Total cell lysates were collected and subjected to a Western blot analysis using the indicated antibodies. (E) Extracellular lactate was detected in HeLa cells which were treated with 10 μM compound C or AMPK-DN, followed by HBSS starvation for 1 h. Data in (B) and (E) were the mean ± S.E.M. from at least three independent experiments. * $p<0.05$, indicating significant effects of HBSS starvation and compound C compared to control cells. # $p<0.05$, indicating significant inhibition of HBSS starvation-induced intracellular ATP loss and lactate formation by compound C and AMPK-DN.

LC3 construct to monitor the maturation process of autophagosomes and autolysosomes. Results showed the significant appearance of red dots (i.e., a signal of autolysosome) and yellow dots (i.e., a signal of autophagosome) in cells receiving HBSS starvation (Fig. S1B), suggesting the ability of HBSS starvation to induce autophagic flux. Next, we determined if PDK activation is involved in HBSS starvation-induced autophagy. To this end, we used DCA to inhibit PDK activity. As shown in Fig. S1C, DCA did not have a significant effect on HBSS starvation-induced LC3 conversion. This result suggests that PDK activity is not involved in HBSS starvation-induced autophagy.

4. Discussion

In addition to hypoxia, starvation is another characteristic of solid tumors. In contrast to hypoxia, the role of starvation in tumorigenesis still remains elusive. So far, starvation-induced autophagy is the most well-known mechanism to promote tumor cell survival [30,31]. In the present study, we tried to elucidate the effects and underlying molecular events of nutrient deprivation on metabolic changes, which thus affect cell viability. Therefore, we used HBSS as a starvation model and found that nutrient deprivation enhances the Warburg effect and delays cell apoptosis induced by HBSS starvation.

The Warburg effect is proven to enhance tumorigenesis and has garnered attention as a target for tumor treatment [32,33]. Lactate, the final product of the Warburg effect, is shown to impact various aspects of tumorigenesis, including immune escape [5,34], cell migration [35,36], and radio-resistance [37,38]. Our present data for the first time unveil a new cascade linking nutrient starvation and Warburg effect. In metabolic profiles, we found that HBSS starvation could induce lactate production in various cancer cell types and even in normal cells. However, in normal cells, Warburg effect exhibits the cell type specificity. Currently the reason why HBSS cannot induce Warburg effect in MEF unlike that seen in HUVEC is not fully understood. Moreover, in HeLa cells HBSS starvation cannot affect glucose uptake, but increases cytosolic pyruvate level, while inhibits oxygen consumption. Notably,

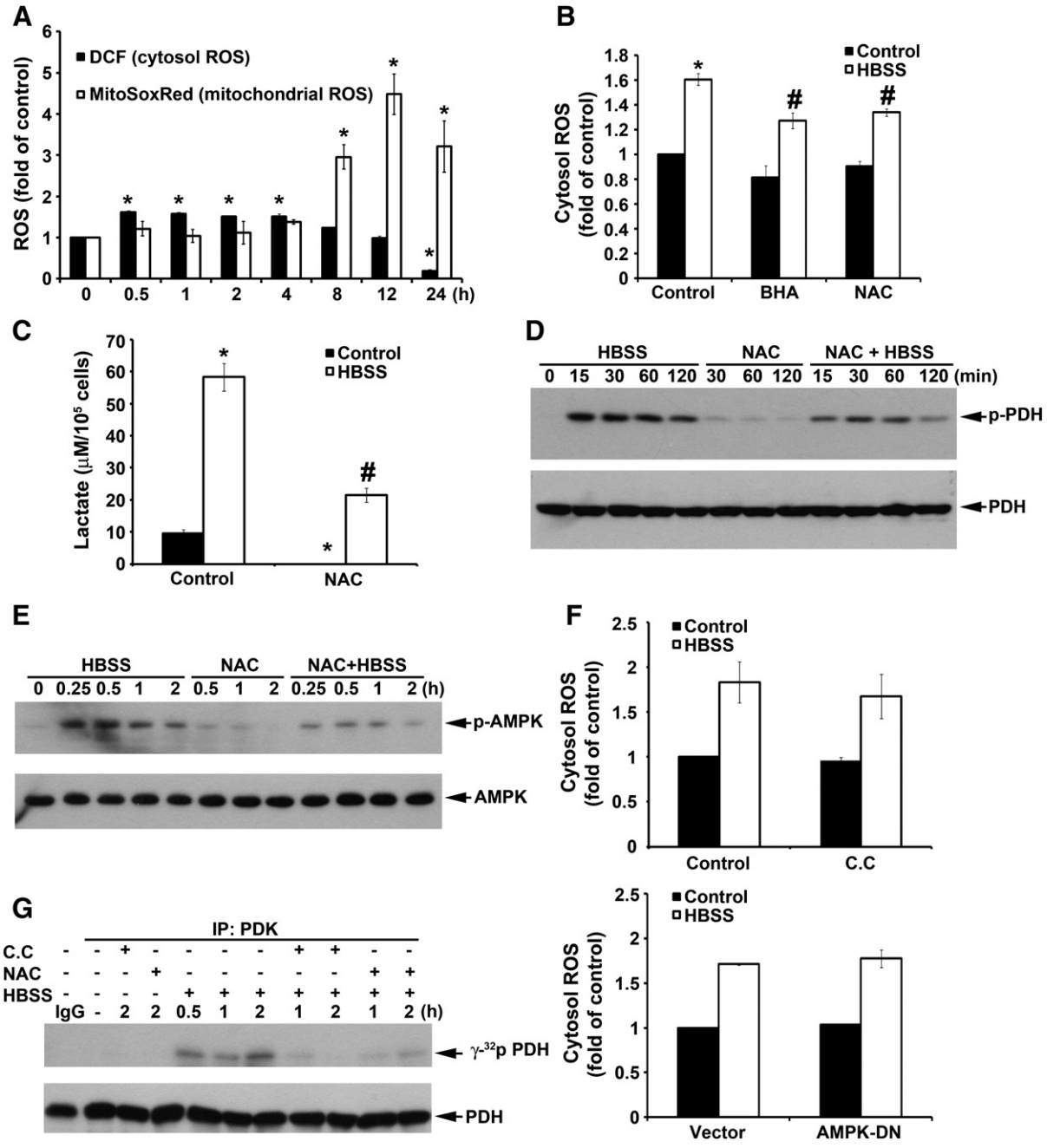

Fig. 4. ROS are involved in the nutrient deprivation-induced Warburg effect and are upstream signals of AMPK. (A) Cytosolic and mitochondrial ROS were detected after HeLa cells were treated with HBSS for the indicated time periods. (B, C) Cytosolic ROS (B) and extracellular lactate (C) were detected after HeLa cells were treated with HBSS for 1 h in the absence or presence of BHA (100 μM) or NAC (10 mM). (D, E) HeLa cells were treated with or without 10 mM NAC for 30 min followed by HBSS starvation for the indicated time periods. Phosphorylation of PDH (D) and AMPK (E) was detected by a Western blot analysis. (F) Cytosolic ROS were detected after HeLa cells were treated with HBSS for 1 h with 10 μM compound C or overexpression of AMPK-DN. (G) After treatment with HBSS with or without 10 μM compound C or 10 mM NAC for the indicated time periods, PDK was immunoprecipitated and subjected to a kinase assay. Data in (A), (B), (C), and (F) were the mean ± S.E.M. from at least three independent experiments. * $p<0.05$, indicating significant effects of HBSS starvation and NAC compared to control cells. # $p<0.05$, indicating significant inhibition of HBSS starvation-induced cytosolic ROS and lactate formation by BHA or NAC.

unlike hypoxia, another typical tumor environment that facilitates the Warburg effect by upregulating enzyme expression of PKM2 and LDH [39,40], we did not observe any changes in LDH activity, PKM2 Tyr105 phosphorylation or PKM2 protein expression after HBSS starvation for 2 h. Nevertheless, similar to hypoxia [41], HBSS starvation also induced PDH phosphorylation and inactivated PDH, leading to the inhibition of the conversion of pyruvate to acetyl-CoA, but the increase of pyruvate metabolism to lactate by LDH. Since PDH condenses the pyruvate into acetyl-CoA in mitochondria, the inhibition of PDH activity by nutrient deprivation should be responsible for the elevated cytosolic pyruvate level. For hypoxia-induced PDH phosphorylation, transcriptional upregulation of PDK expression via the HIF-1α pathway is suggested [42]. On the other hand, long-term starvation for 48 h was also reported to induce PDH phosphorylation by upregulation of PDK expression in the rat heart and skeletal muscle [42,43]. In this aspect, we did not detect any changes in PDK expression in HeLa cells undergoing HBSS starvation within 2 h. In our case, however, we found that starvation increases PDH phosphorylation via PDK activation. This notion is supported by the parallel and rapid increases in PDK activity

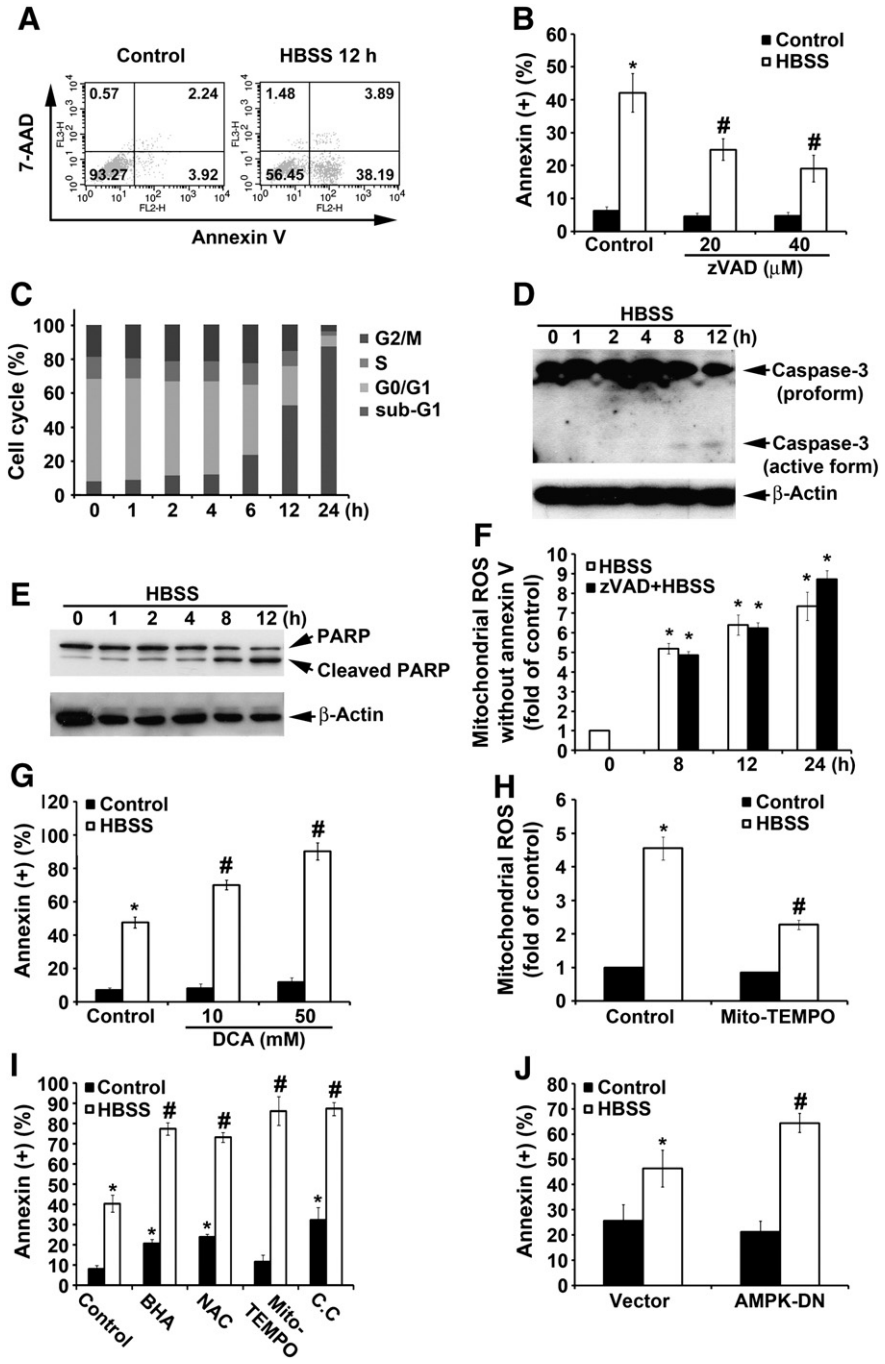

Fig. 5. The Warburg effect reduces cell apoptosis upon nutrient deprivation. (A, B) After treatment with HBSS for 12 h with or without zVAD, cells were collected for the cell death analysis using Annexin V/7-AAD double staining. (C) Cell cycle analysis using propidium iodide (PI) staining was conducted after HBSS starvation for the indicated time periods. (D) Caspase-3 activation and (E) PARP cleavage were detected by a Western blot analysis after HBSS starvation for the indicated time periods. (F) After treatment with HBSS for 8–24 h with or without zVAD, cells were stained with MitoSoxRed and annexin V. (G, I, J) After treatment with HBSS for 12 h with or without DCA (G), BHA (100 μM), NAC (10 mM), Mito-TEMPO (500 μM), compound C (10 μM) (I), or overexpression of AMPK-DN (J), cells were collected for a cell death analysis using Annexin V/7-AAD double staining. (H) After treatment with HBSS for 12 h with or without Mito-TEMPO (500 μM), cells were collected for mitochondrial ROS analysis using MitoSoxRed. Data in (B), (F)–(J) were the mean ± S.E.M. from at least three independent experiments. * $p<0.05$, indicating significant effects of HBSS starvation and the indicated agents compared to control cells. # $p<0.05$, indicating significant inhibition of HBSS starvation-induced responses by various manipulations.

and PDH phosphorylation after nutrient starvation, as well as the inhibitory effect of DCA in this event.

HBSS starvation also concomitantly and rapidly reduces intracellular ATP content and induces AMPK activation within 15 min. Since an increased AMP/ATP ratio induces AMPK phosphorylation, the reduced ATP level is at least one of the possibilities for inducing AMPK activation in cells subjected to HBSS conditioning. Moreover, besides ATP loss, we also showed the involvement of cytosolic ROS in AMPK activation at least at the early stage of HBSS starvation. The ROS scavenger NAC significantly inhibited HBSS starvation-induced AMPK phosphorylation, while the AMPK inhibitor had no significant effect on HBSS starvation-induced cytosolic ROS. Consistently, ROS were found to be upstream molecules of AMPK [44,45]. Regarding cytosolic ROS increase, we suggest that it is generated from NADPH oxidase, because previous studies showed that serum starvation and growth factor starvation induced ROS production through NADPH oxidase [46,47]. Moreover, aberrant ROS generation initially occurring through NADPH oxidase was reported to facilitate mitochondrial damage [48]. Therefore,

we suggest that the early cytosolic ROS production caused by HBSS starvation might lead to remarkable ROS production from mitochondria at late stage. Moreover, under our examination period for 2 h, the time course of cytosol ROS production is correlated with AMPK phosphorylation. Thus we suggest that both ATP loss and cytosolic ROS production might coordinately mediate the HBSS starvation-induced AMPK pathway. Nevertheless, we still cannot rule out the possible regulation between AMPK and mitochondrial ROS at late stage of HBSS starvation and subsequent outcomes in terms of Warburg effect and cell death, for example after 8 h upon an apparent mitochondrial ROS being increased.

Previous studies showed the ability of AMPK to induce glycolysis via activation of PFK2 [21,22]; however, the metabolic outcome in terms of the Warburg effect remains unknown. In this study, we found that the intracellular pyruvate level after short-term treatment with HBSS starvation is not changed, but we did detect an increased cytosolic pyruvate level, which might contribute to the rapid production of lactate. In this study, we for the first time demonstrated the involvement of ROS-dependent AMPK in PDK activation. Due to significant inhibition of PDH phosphorylation by compound C, NAC, and expression of AMPK-DN, we suggest that ROS production and AMPK activation induced by HBSS starvation mediate PDH phosphorylation. In agreement with these findings, NAC and compound C can reduce PDK activity.

Since PDH is a key enzyme controlling pyruvate catabolism by shifting pathways between mitochondrial phosphorylation and LDH formation; molecules which can modulate PDH phosphorylation should have an effect on pyruvate metabolism. In this aspect, NAC was shown to improve mitochondrial TCA metabolism by stimulating carbon flux through PDH, while the underlying molecular event has never been elucidated [49]. Our current results not only support previous findings, but also highlight the role of ROS in shifting energy-producing processes from mitochondrial metabolism to the Warburg effect. We showed that NAC treatment alone in normal medium can alter the Warburg effect and mitochondrial metabolism in a reverse manner, i.e., reducing lactate formation but increasing oxygen consumption (data not shown). Likewise, HBSS starvation-induced lactate production is significantly inhibited by NAC. Similar to NAC, cysteine significantly inhibits HBSS-induced PDH phosphorylation. It may be because cysteine is a precursor of glutathione and possesses the antioxidant activity [50]. AMPK was shown to exert multiple effects on metabolic changes, and in the present study, we demonstrated that HBSS starvation-induced AMPK activation led to PDH phosphorylation and lactate production. However, we also observed that compound C itself induced moderate PDH phosphorylation without affecting PDK activity. Currently we cannot provide explanation for this discrepancy, and the effects of compound C on PDH phosphatase and/or other unidentified kinases of PDH still need to be investigated in the future. Therefore, we for the first time demonstrated that AMPK controls not only the glycolysis pathway by phosphorylation of PFK2 [21,22] but also a pyruvate metabolic shift by regulation of PDK activity. Furthermore, since PDK is a mitochondrial protein and AMPK locates in cytosol, we speculate that the AMPK-dependent regulation of PDK occurs in an indirect manner.

Starvation was shown to induce apoptosis in different cell types [51], and our results also support this notion. In our study, HBSS starvation induces apoptosis and the active form of caspase-3 is induced in a time course correlated with PARP-1 cleavage, an index of caspase-3 activation. Furthermore, we found that inhibition of the Warburg effect by DCA enhances HBSS starvation-induced cell death. Inhibition of PDK upstream molecules, ROS and AMPK, produces similar results as with DCA. These data suggest that an increased Warburg effect upon HBSS starvation confers greater resistance to the death process upon cells. Besides the Warburg effect, autophagy is an evolutionarily conserved phenomenon for maintaining homeostatic functions such as protein degradation and organelle turnover. Nutrient deprivation was shown to induce autophagy which can delay nutrient deprivation-induced cell death [52]. In the present study, autophagy indeed is induced upon HBSS starvation; however, our data using DCA rule out the involvement of PDK in autophagy induction in the case of nutrient deprivation.

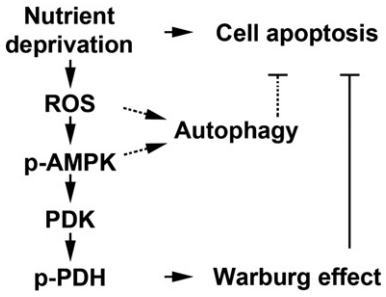

Fig. 6. Schematic summary of the nutrient deprivation-induced Warburg effect and autophagy in HeLa cells. HBSS starvation induces the cytoprotective Warburg effect by upregulating the PDK activity via an AMPK-dependent mechanism. The ROS-mediated AMPK pathway leads to PDK activation and PDH phosphorylation, which in turn loses its ability to convert pyruvate to acetyl-CoA, but increases pyruvate metabolism to lactate by LDH. In addition, nutrient starvation also induces autophagy, which provides an additional pathway independent of Warburg effect to protect cells against metabolic stress.

The Warburg effect provides several growth advantages for cancer cells. Hypoxia is well known to induce the Warburg effect through HIF-1α accumulation. In the present study, we demonstrated that nutrient deprivation, another characteristic of solid tumors, also induces the Warburg effect to support cell viability upon starvation stress (Fig. 6). Unlike HIF-1α which induced expression of PDK, we for the first time demonstrated that a low-nutrient condition drives cancer cells to utilize glycolysis to produce ATP, and this increased Warburg effect is through a novel mechanism involving ROS/AMPK-dependent activation of PDK. In addition, autophagy provides an alternative mechanism in accompany with Warburg effect to protect cells against nutrient stress.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2013.01.025.

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